High Frequency of Carcinogen-induced Early, Preneoplastic Changes in Rat Tracheal Epithelial Cells in Culture

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ABSTRACT

To study the mechanisms of carcinogenesis, we have developed a system that uses normal cells from an environmentally and epidemiologically relevant tissue, respiratory epithelium. The induction of preneoplastic variants of epithelial cells in culture was quantitated on a per-cell basis following exposure of rat tracheal epithelial (RTE) cells in vitro to the direct-acting carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Following treatment of normal RTE cells, large colonies of altered cells exhibiting an enhanced growth potential under selective culture conditions were observed, while normal RTE cells ceased proliferation after several cell doublings. After further growth in culture, these altered cells acquired the ability to grow in semisolid medium and to produce squamous cell carcinomas when injected into nude mice. The induction of enhanced growth variants of RTE cells by MNNG occurred with a high frequency (>2.6%/colony-forming cell). In addition, a linear dose-response curve with a slope of ~1 was observed when the logarithm of MNNG-induced transformation frequency was plotted versus the logarithm of MNNG dose. These results are consistent with a one-hit mechanism for induction of preneoplastic variants of RTE cells by MNNG. Similar frequencies and kinetics of induction of preneoplastic variants in other culture systems using diploid cells have been observed, suggesting a common mechanism for this early step in carcinogenesis. The RTE cell system will be useful for mechanistic studies of early as well as late changes in the development of neoplasia by epithelial cells.

INTRODUCTION

The use of cell culture systems to study neoplastic transformation has provided important information on the cellular and molecular basis of carcinogenesis (4, 12). Cell culture systems using cells from the predominant target tissues for environmental carcinogens and human cancers, i.e., epithelial cells, are the most relevant. Unfortunately, most quantitative cell transformation assays use fibroblasts (2), although malignant tumors of nonepithelial cells account for only 10 to 20% of human malignant neoplasms. While recent advances have been made (8, 11,15, 16, 26, 28, 29), there is still a great need for epithelial cell culture systems for quantitative studies of carcinogenesis.

We have developed a quantitative system for studying the early as well as late changes in epithelial cell transformation. The target cells for this model are RTE4 cells in culture, because respiratory epithelium is an important site for environmental carcinogens in humans. Furthermore, in vivo models for carcinogenesis with this tissue are established (21) and, hence, comparative studies in vivo and in vitro can be performed.

The important observation by Marchok et al. (19, 20), that one could select for carcinogen-induced preneoplastic tracheal cells by their growth properties in vitro, led to quantitative studies on the cellular alterations that occur during neoplastic development in RTE in vivo. Tracheas exposed to carcinogens contained epithelial cells with an increased capacity for growth in vitro. Furthermore, these altered cells were specifically found in sections of tracheas which have moderate to severe atypia by conventional cytological and histopathological criteria (18). These altered cells were shown to progress to malignant cells following further growth in vitro forming squamous cell carcinomas when injected into syngeneic animals (13). Terzaghi and Nettlesheim (30) used this in vitro selection technique for carcinogen-altered cells to quantitate the cellular changes occurring in tracheal epithelium in vivo during the process of carcinogenesis.

Carcinogenesis studies of RTE cells can also be carried out in vitro using culture conditions developed in our laboratory for the growth of normal RTE cells (10, 33). Using these culture conditions, we have been able to quantitate the cytotoxic response of normal RTE cells to a variety of carcinogens (10). For our transformation assay, normal RTE cells are exposed to carcinogens in vitro using culture conditions which are permissive for the growth of only preneoplastic and neoplastic RTE cell variants having an enhanced growth potential in vitro. These altered cells, termed EG variants, are scored as colonies of proliferating cells at the end of the selection period (4 to 6 weeks). The frequency of carcinogen-induced altered cells can be calculated from the number of RTE cells surviving treatment and the number of EG variants. This assay enables us to perform quantitative carcinogenesis studies with normal RTE cells in vitro.

Pai et al. (23) demonstrated that neoplastic transformation of RTE cells in culture could be induced by exposure to MNNG in vitro. These authors were able to quantitate population changes in the carcinogen-treated cultures 18 days after treatment and estimated that the frequency of carcinogen-induced preneoplastic cells was ~10^-2 per surviving cell.

The present study provides (a) a precise determination of the frequency of MNNG-induced EG variants of RTE cells in culture per colony-forming cell; (b) a determination of whether the induction is consistent with a one-hit mechanism; and (c) attempts to determine the time required for the expression of this alteration following carcinogen treatment.

Studies of cellular transformation in fibroblasts have led to the surprising result that, in some cell culture systems, chemical...
carcinogens induce a high frequency (>1%) of altered cellular phenotypes associated with preneoplastic or neoplastic states (2, 6). This finding has led some investigators to question the somatic mutation theory of carcinogenesis (6), although certain mutational mechanisms are compatible with these results (1). It is unknown whether the same frequency of carcinogen-induced changes observed in fibroblasts occurs also in epithelial cells, from which most human cancers arise. The frequencies of occurrence of early events in the neoplastic development of epithelial cells reported here can, thus, be compared to the frequencies of similar changes in fibroblasts in culture. This comparison will be important for studies on the mechanism of carcinogenesis.

A demonstration that the induction of preneoplastic changes in cells occurs by a one-hit mechanism (9) is important for several reasons. (a) A one-hit mechanism of induction suggests that only a single event in the induction of these altered cells is affected by carcinogen treatment. A determination of the role of carcinogens in the induction of preneoplastic alterations in cells is one crucial aspect needed for understanding the mechanism of induction of cancer. (b) A one-hit mechanism of induction of altered cells is useful for assessing the risk of various low doses of carcinogens. The demonstration that a single step in the carcinogenesis process follows one-hit kinetics is of theoretical importance for development of models for carcinogen low-dose extrapolation. (c) Comparisons can be made with preneoplastic changes in different cell systems and with mutagenic processes in cells which also occur by one-hit mechanisms in order to determine the similarities or differences in these processes which may improve our overall understanding of the development of neoplasia.

For these reasons, the results of this study are important in order to understand the mechanism of early, preneoplastic changes in epithelial cells. Furthermore, these studies, in addition to earlier reports from our laboratory (10, 23, 33), provide the basis for a quantitative system for studying neoplastic progression of epithelial cells in culture.

MATERIALS AND METHODS

RTE Cell Culture. RTE cells were obtained from 8-week-old male Fischer 344 rats (specific-pathogen-free). Animals were sacrificed by CO2 asphyxiation, and their tracheas were surgically exposed. The proximal ends were intubated with flanged 1.14 (inside diameter) x 1.57-mm (outside diameter) polyethylene tubing (Clay Adams, Parsippany, N. J.) and secured with Size 00 silk (Ethicon, Somerville, N. J.). Tracheas were removed and washed with Ham's F-12 (Grand Island Biological Co., Grand Island, N. Y.) plus antibiotics (see below), and a 1% Pronase solution (Type VI; Sigma Chemical Co., St. Louis, Mo.) in Ham's F-12 was slowly infused into each trachea through the tubing. The distal ends were ligated, the tracheas were filled to extension with Pronase solution, and the tubing was clamped. The tracheas were incubated for 1 to 1.5 hr at room temperature in Ham's F-12. Following incubation, 10 ml of growth medium, consisting of Ham's F-12, 5% fetal bovine serum (Grand Island Biological Co.), insulin (1.0 μg/ml; Sigma), hydrocortisone (0.1 μg/ml; Sigma), penicillin (100 units/ml), and streptomycin (100 μg/ml; Grand Island Biological Co.), were slowly infused through each trachea after the distal end had been severed just proximal to the tracheal. RTE cells detached by Pronase digestion were collected after passing the suspension through a 100-μm gauze (Tetro, Inc., Elmsford, N. Y.) to remove cell clumps. The RTE cell suspension was centrifuged for 10 min at 150 × g and resuspended vigorously in growth medium, the cell number was determined using a hemacytometer, and 102 to 103 cells were plated into dishes containing lethally irradiated 3T3-J2 fibroblasts (feeder cells) (24, 25) (see below). Cultures were incubated at 37°C in a humid atmosphere of 5% CO2 and 95% air.

Feeder Cell Preparation. 3T3-J2 fibroblasts (gift from Dr. H. Green) were grown to near confluence in 75-sq cm flasks (Falcon Plastics, Oxnard, Calif.) at 37°C, in a moist atmosphere of 10% CO2 and 90% air in Dulbecco's modified Eagle's (Grand Island Biological Co.) medium with 10% fetal bovine serum. To prepare feeder cells, 3T3 cells were treated with a 0.1% trypsin solution for 5 min at 37°C, and washed with Ham's F-12 medium plus 5% fetal bovine serum, resuspended in the same medium, and irradiated with 5000 rads from a 137Cs source (J. S. Shepard Assoc., Glendale, Calif.). The cells were resuspended in the above medium, plated in 2-ml aliquots at 1 to 2 x 105 cells/sq cm (1 to 2 x 105 cells/35-mm dish), and allowed to attach overnight.

Cytotoxicity Assay. Twenty-four hr after plating RTE cells [before any cell division occurred (10)], cultures were exposed to MNNG (Chemical Repository, National Cancer Institute, Bethesda, Md.) in growth medium for 4 hr. Stock solutions of MNNG were prepared in dimethyl sulfoxide (silylation grade; Pierce Chemical Co., Rockford, Ill.) or in distilled water. Carcinogen-induced cytotoxicity was measured as the number of cells that survived MNNG treatment and proliferated to form colonies (>50 cells). Seven days after carcinogen exposure, 5 dishes from each treatment group were selected randomly, and these cultures were fixed with methanol and stained with 10% aqueous Giemsa, and the number of colonies per dish was scored. Relative survival was determined by comparing the colony-forming efficiency of the MNNG-exposed groups to that of the control treatment group.

Transformation Assay. One week following MNNG treatment of RTE cell cultures, 3T3 feeder cells were removed from the dishes, and RTE cells were either left in place for 4 to 5 weeks, as described below, or were subcultured into dishes without feeder cells. 3T3 feeder cells were removed by washing the cultures with Ca2+-, Mg2+-free phosphate-buffered saline, followed by a 3- to 5-min treatment with EDTA (0.002%) and vigorous pipetting. RTE cell colonies were then dissociated with a 0.05% trypsin:0.02% EDTA (Grand Island Biological Co.) solution for 5 min at 37°C. Two ml of medium of growth medium was added to each dish and pipetted vigorously, and the cell suspension was transferred to a new dish. Growth medium was changed weekly for an additional 4 to 5 weeks, and cultures were then fixed and stained. Transformed colonies were scored 5 to 6 weeks after carcinogen treatment as large colonies (>100 cells) of small hyperchromatic cells with increased nuclear to cytoplasmic ratios (see Fig. 1 and "Results").

Quantitation of Transformation Frequency. The transformation frequency was determined by one of 2 methods. The first was simple enumeration of the EG variants per total number of colony-forming cells surviving treatment. Alternatively, the frequency of EG variants was determined based on the Poisson distribution from the number of dishes containing no transformed cells using:

\[ P_n = e^{-n} \]

where \( P_n \) is the fraction of dishes without transformed colonies, and \( n \) is the average number of transformation events per dish, and

\[ \% \text{ transformation frequency per colony-forming cell} = \left( \frac{n}{CFC} \right) \times 100 \]

where CFC is colony-forming cells per dish (i.e., cells plated per dish, multiplied by colony-forming efficiency in solvent-treated control, multiplied by surviving fraction).

Establishment of EG Variant Cell Lines. Dishes containing large EG variants were identified, and these colonies were allowed to reach 1 to 2 cm in diameter. Contaminating fibroblasts, if present, were selectively removed by a brief (2-min) treatment with 0.05% trypsin:0.02% EDTA (Grand Island Biological Co.) followed by a vigorous wash with phosphate-buffered saline. EG variant cells were then subcultured by treatment for 5 additional min with the same trypsin:EDTA solution. EG variants were replated into dishes either with growth medium only or with 3T3 feeder cells. Subsequent subcultures were made without feeder cells at weekly intervals using 1:10 to 1:20 dilutions.

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Anchorage Independence and Tumorigenicity Tests. EGV cell lines were tested for anchorage-independent growth in agarose medium (Sea Plaque agarose; Marine Colloids, Rockland, Maine), as described in Ref. 32, using a 0.6% agarose base, 0.3% agarose top layer, 20% fetal bovine serum, and growth medium. EGV cell lines were tested for their tumorigenic potential, as described in Ref. 32, by injecting 2 × 10⁶ cells s.c. into nude mice [Crl:nu/nu (CD-1/Br), Charles River Laboratories, Wilmington, Mass.). Tumors were removed, fixed in Bouin’s fixative, dehydrated, and embedded in paraffin. Histological sections were stained with hematoxylin and eosin.

RESULTS

Characterization of EG Variants in RTE Cell Cultures. As originally described by Marchok, et al. (19, 20) and Terzaghi and Nettelsheim (30), carcinogen treatment of rat tracheas in vivo induces heritably altered cells which have the capacity to proliferate in vitro under conditions where normal RTE cells fail to grow. We have termed these altered cells EG variants. In contrast, normal RTE cells do not divide more than once or twice when plated in standard tissue culture medium alone in the absence of 3T3 feeder cells (10) or 3T3 cell conditioning factors (33). Normal RTE cells remain on dishes from which 3T3 feeder cells have been removed (conditioned dishes), they proliferate for 1 to 2 weeks, and then their rate of proliferation decreases and is eventually undetectable. Normal RTE cells grown under these nonpermissive conditions also begin to enlarge and appear squamous-like as their rate of proliferation decreases. The cells either detach from the dish after 1 to 2 weeks or persist in the culture for longer periods of time without cell division. This process appears analogous to the process of terminal differentiation observed in cultures of rodent epithelial cells (27, 34, 35). Normal RTE cells remaining after 6 to 10 weeks in culture could not be subcultured even onto 3T3 feeder cells.

Under our experimental conditions, EG variants were recognized at 4 to 6 weeks after treatment of primary cultures of RTE cells with MNNG as large colonies of proliferating epithelial cells which were small, had an increased nuclear to cytoplasmic ratio, and appeared hyperchromatic after staining (Fig. 1). EG variants were seen in untreated control cultures (see below) with frequencies ranging from <0.02 to 0.2%, and these were morphologically indistinguishable from MNNG-induced EG variants. EG variants could also be subcultured. When 3T3 feeder cells were included in secondary cultures to improve colony-forming efficiencies, 40 of 45 EG variants were subculturable. Subsequent passages of EG variants were performed without feeder cells.

Cell lines established from 10 EG variants were tested for growth in agarose and tumorigenicity in nude mice beginning 5 passages after their isolation. Initially, EG variant cell lines were anchorage-dependent or weakly anchorage-independent (<0.01% colony-forming efficiency in agarose medium) and were nontumorigenic, with no tumors observed at 4 to 6 months after s.c. injection of 2 × 10⁶ cells into nude mice. At subsequent passages, most EG variant cell lines became anchorage-independent (colony-forming efficiency ≥1% in agarose medium), but were nontumorigenic until later passages (>20 passages). Two of 10 EG variant cell lines were tumorigenic as early as passage 6 with latent periods of 5 to 12 weeks. Both lines yielded tumors classified as squamous cell carcinomas (Fig. 2).

Quantitation of EG Variants in RTE Cell Cultures. RTE cells grown on 3T3 feeder cells were treated with solvent only or with various concentrations of MNNG for 4 hr. The relative survival for each dose of MNNG was determined from a group of 5 dishes fixed and stained 1 week after treatment. The colony-forming efficiency of the solvent-treated cells was 6.6% and, following treatment with MNNG, a logarithmic decrease in cell survival was observed (Table 1).

A dose-dependent increase in the frequency of EG variants was observed following treatment of the cells with MNNG (0.1 to 1.0 μg/ml) (Table 1). The transformation frequency was calculated as the number of EG variants per colony-forming cell surviving treatment (determined from MNNG-induced cytotoxicity). After treatment with MNNG (1.0 μg/ml), which resulted in a 50% relative survival of the cells, the frequency of EG variants was 2.9% of the surviving cells. This is 16-fold greater than the frequency of EG variants in control cultures, which was 0.18% under these conditions. Although there is some fluctuation in the frequency of spontaneous and induced EG variants between experiments (for example, see Chart 2), the frequency of MNNG-induced EG variants always increases with increasing doses of MNNG from 0.1 to 1.0 μg/ml, yielding maximum increases of 10 to 50-fold above background. When the dose-response of MNNG-induced transformation was plotted as the logarithm of dose, a linear dose-response curve was observed with a slope of 1.12 (Chart 1).

Calculation of Transformation Frequency by P₀ Method. The frequency of occurrence of carcinogen-induced preneoplastic RTE cells was determined from the number of dishes without transformed cells (17). The frequency of altered cells was then calculated based on the Poisson distribution using $P₀ = e^{-n}$ where $P₀$ is the fraction of dishes without transformed colonies, and $n$ is the average number of transformation events per dish. The transformation frequency per colony-forming cell was equal to $n$/CFC, where CFC is colony-forming cells per dish (i.e., colony-forming efficiency multiplied by surviving fraction). The transformation frequencies obtained using the $P₀$ method of calculation were the same as those obtained based on the number of EG variants per colony-forming cell (Table 1). As above, a linear dose-response curve with a slope of 1.12 was obtained when the logarithm of cell transformation frequency was plotted versus the logarithm of dose (Chart 1).

Distribution of EG Variants. The distribution of EG variants was analyzed. The number and frequency of occurrence of EG...
Neoplasia Transformation of Tracheal Epithelial Cells

Table 1

<table>
<thead>
<tr>
<th>MNNG Concentration (µg/ml)</th>
<th>No. of dishes</th>
<th>Colony-forming cells/dish</th>
<th>EG variants/dish</th>
<th>% transformation frequency based on EG variants</th>
<th>% transformation frequency based on Po(0)</th>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>60</td>
<td>66 ± 1.3</td>
<td>0.12 ± 0.04</td>
<td>0.18 ± 0.06</td>
<td>53/60</td>
</tr>
<tr>
<td>0.1</td>
<td>60</td>
<td>55 ± 1.6</td>
<td>0.12 ± 0.05</td>
<td>0.22 ± 0.09</td>
<td>54/60</td>
</tr>
<tr>
<td>0.5</td>
<td>60</td>
<td>49 ± 1.6</td>
<td>0.72 ± 0.11</td>
<td>1.47 ± 0.24</td>
<td>31/60</td>
</tr>
<tr>
<td>1.0</td>
<td>59</td>
<td>35 ± 1.6</td>
<td>1.02 ± 0.14</td>
<td>2.91 ± 0.43</td>
<td>25/59</td>
</tr>
</tbody>
</table>

* 10^6 plated cells/dish.

** EG variants/dish/colony-forming cells/dish x 100

S.D. of the ratio = \( \sqrt{\frac{\chi^2}{\chi^2}} - \left[ \frac{\chi^2}{\chi^2} \right] \)

\( \frac{P_0}{n} = \text{No. of dishes with } n \text{ EG variants/Total no. of dishes} \)

\( \% \text{ transformation frequency} = \frac{n}{\text{colony-forming cells per dish}} \times 100. \)

\( P_0 = e^{-n} \)

where \( n \) is the average number of transformation events per dish.

* Dimethyl sulfoxide treatment only, 0.1%.

\( \bar{S.D.} \text{ of the mean} \)

Table 2

Comparison of observed and expected occurrences of EG variants based on the Poisson distribution

The distribution of EG variants in the dishes used to calculate the transformation frequencies in Table 1 was determined. The number of EG variants per dish was determined for each dose of MNNG, and the distribution of those EG variants was tabulated as shown (Observed). The expected frequency of EG variants per dish was determined from the Poisson distribution using the mean number of EG variants per dish. The calculated distribution of EG variants is shown (Expected). The index of dispersion was determined for each dose as described in the text, and the significance of the statistic was tested using the \( \chi^2 \) distribution with \( (N - 1) \) degrees of freedom.

<table>
<thead>
<tr>
<th>MNNG concentration (µg/ml)</th>
<th>No. of dishes with n EG variants/dish</th>
<th>Variance</th>
<th>Significance of deviation from Poisson distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 0</td>
<td>n = 1</td>
<td>n = 2</td>
</tr>
<tr>
<td>0.0 µg/ml</td>
<td>Observed</td>
<td>53</td>
<td>7</td>
</tr>
<tr>
<td>Expected</td>
<td>53.4</td>
<td>6.2</td>
<td>0.4</td>
</tr>
<tr>
<td>0.1 µg/ml</td>
<td>Observed</td>
<td>54</td>
<td>5</td>
</tr>
<tr>
<td>Expected</td>
<td>53.4</td>
<td>6.2</td>
<td>0.4</td>
</tr>
<tr>
<td>0.5 µg/ml</td>
<td>Observed</td>
<td>31</td>
<td>18</td>
</tr>
<tr>
<td>Expected</td>
<td>29.3</td>
<td>21.0</td>
<td>7.5</td>
</tr>
<tr>
<td>1.0 µg/ml</td>
<td>Observed</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>Expected</td>
<td>21.7</td>
<td>22.1</td>
<td>11.2</td>
</tr>
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Effect of Selective Pressure on RTE Cell Transformation. The effect on the induced and spontaneous transformation frequencies of imposing selective growth conditions at various times after carcinogen exposure was examined to gain insight into the time required for expression of carcinogen-induced events. This was accomplished by either selective removal of the feeder cells from the culture, as described in "Materials and Methods," or by feeder removal plus subculturing the cells. These methods affected the growth of normal RTE cells, but not of EG variants. Unfortunately, neither method immediately blocked the proliferation of the normal cells, which continued to divide for 4 to 7 days after the selective pressure was imposed. Subculturing inhibited cell proliferation more than feeder cell removal only.

Following MNNG treatment, similar increases in the frequency of EG variants with respect to dose were observed in cultures with or without selective pressure at Day 4 or 7 (Chart 2). Similar induced transformation frequencies were observed for cultures without selection, with feeder removal only, or with feeder removal and subculturing. Selection at Day 4 by feeder removal or at Day 7 by subculturing resulted in slightly lower induced
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Chart 2. Frequency of transformation of RTE cells to EG variants versus MNNG dose. Selection for EG variants was applied, as described in "Materials and Methods" and "Results," on the days indicated. Transformation frequencies were calculated by the Poisson method.

Table 3

<table>
<thead>
<tr>
<th>MNNG dose (µg/ml)</th>
<th>No. of cells/dish (x 10^5)</th>
<th>No. of colony-forming dishes</th>
<th>P0</th>
<th>% of transformation frequency based on P0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>55 ± 4.3^a</td>
<td>18/20</td>
<td>0.19</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>164 ± 3.5</td>
<td>17/19</td>
<td>0.07</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>302c^c</td>
<td>17/18</td>
<td>0.02</td>
</tr>
<tr>
<td>0.3</td>
<td>2</td>
<td>12 ± 2.1</td>
<td>18/20</td>
<td>0.86</td>
</tr>
<tr>
<td>0.3</td>
<td>5</td>
<td>48 ± 4.0</td>
<td>8/14</td>
<td>1.17</td>
</tr>
<tr>
<td>0.3</td>
<td>10</td>
<td>79^c^c</td>
<td>6/18</td>
<td>1.39</td>
</tr>
</tbody>
</table>

^a Cultures were exposed to MNNG in Ham’s F-12 buffered with 0.02 w/v 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 6.8) for 4 hr.

Effect of number of cells at risk on transformation frequency. The spontaneous transformation frequency was ≤0.05% if selection was applied by feeder removal at Day 4 or by subculturing at Day 4 or 7, compared to 0.2% in cultures with no feeder removal or subculture.

Effect of Cell Number on RTE Cell Transformation. The effect of cell number per dish on the transformation frequency was examined following feeder cell removal on Day 7. The frequency of transformation on a per-cell basis following treatment with MNNG (0.3 µg/ml) was independent of the number of cells treated (Table 3). Treatment of increasing numbers of cells resulted in an increase in the number of transformed cells and, hence, a decrease in the P0 value. However, the transformation frequencies calculated by the P0 method at all cell densities were very similar (0.9 to 1.4%). The transformation frequency in untreated cultures was slightly higher (0.2%) at the lowest cell density than at higher cell densities (0.02 to 0.07%).

DISCUSSION

We have analyzed the induction of EG variants in RTE cell cultures. There are several lines of evidence which indicate that the tracheal cell variants in our studies are preneoplastic: (a) EG variants can be induced by carcinogen treatment of RTE cells in culture or tracheas in vivo (19, 23). (b) Dysplastic sections of tracheas exposed to carcinogens in vivo contain these altered cells, whereas morphologically normal areas of exposed tracheas do not (18). (c) Cells line derived from EG variants form metaplastic or dysplastic lesions when allowed to repopulate denuded rat tracheas in vivo (31, 33). (d) EG variants have an increased propensity to progress in vitro to cells capable of producing squamous cell carcinomas or adenocarcinomas in vivo (20, 23, 30). In our studies, EG variants acquired the ability to grow in agarose and to form tumors in as few as 6 passages in culture.

The basis for the preneoplastic alteration of RTE cells, which is expressed as an enhanced growth potential, is unknown. EG variants may have an increased proliferative capacity, a decreased potential for differentiation, or a combination of these changes. Kulesz-Martin et al. (16) have reported a high frequency (~1%) of carcinogen-altered mouse epidermal cells which are altered in their differentiation capacity, suggesting that changes in differentiation may be important as early, preneoplastic changes.

The induction of EG variants by the direct-acting carcinogen MNNG appears to occur by a one-hit mechanism (9). The general features of a mechanism consistent with the one-hit induction of cellular transformation include the following (9): a Poisson distribution of the induced variants; a linear dose-response curve with a slope of one when the logarithm of transformation frequency is plotted against the logarithm of dose; and a transformation frequency per cell, independent of the number of cells treated. Following treatment of RTE cells with MNNG we observed: a Poisson distribution of induced EG variants (Table 2); a linear dose-response with a slope of 1.12 from a plot of log (transformation frequency) versus log (MNNG dose) (Chart 1); and that the frequency of induction of EG variants was independent of the number of cells treated (Table 3). In addition, EG variants are more sensitive than primary RTE cells to the cytotoxic effects of MNNG (data not shown), suggesting that EG variants do not simply arise by selection of cells which preferentially survive MNNG treatment. Thus, our results are consistent with the hypothesis that EG variants are induced by MNNG and that the induction occurs by a one-hit mechanism.

As indicated in "Introduction," the demonstration that the induction of EG variants occurs by a one-hit mechanism is important for several reasons. A one-hit mechanism suggests that only a single event in the induction of EG variants is affected by MNNG treatment. In addition, it enables comparisons to be made with the results of cell transformation in other cell systems. For example, the induction of morphological transformation of Syrian hamster embryo cells by carcinogens also seems to occur by a one-hit mechanism (9). In contrast, the induction of morphological transformation of C3H 10T1/2 cells seems to occur by a 2-step mechanism (2, 7, 14). It is important to note that the RTE cell system and the Syrian hamster embryo cell system use either freshly isolated or early passage cells and measure the induction of preneoplastic cells. In contrast, the 10T1/2 cell system is based on an established, aneuploid cell line, which has been described as preneoplastic (2). The difference in the transformation process of these different cells may indicate a difference between the mechanism of induction of preneoplastic cells to neoplastic cells and the induction of normal cells to preneoplastic cells. Further studies are needed particularly on the later steps of progression to neoplasia. However, the similarity in the 2 systems (RTE and Syrian hamster embryo cells), measuring conversion of normal cells to preneoplastic cells, suggests a common mechanism for this early step in carcinogenesis.

The induction of early, preneoplastic changes of RTE cells (and Syrian hamster embryo cells (5)) by MNNG occurs with a high frequency (>1%/surviving cell). Any mechanism describing the induction of EG variants must account for this high frequency, and it is, therefore, important to determine the accuracy of these
frequencies by eliminating possible sources of error in their calculation. In addition, it is important to determine the sensitivity and reproducibility of the measurement of preneoplastic transformation of RTE cells, since this model represents an important system for quantitative studies of epithelial cell transformation. Thus, we calculated the frequencies of EG variant induction in 2 ways.

We calculated the frequency of induced EG variants per colony-forming cell from the total number of transformed colonies. This method is subject to the following potential sources of error. If the formation of secondary colonies occurs relatively frequently from primary colonies of EG variants, or if colonies merge, this method will overestimate or underestimate the transformation frequency. Therefore, we also calculated the transformation frequency based on the number of dishes with no EG variants, which is termed the $P_0$ method (17). Use of this method requires that the occurrence of EG variants follows a Poisson distribution, which is the case (Table 2). Use of this method also eliminates possible errors caused by secondary colony formation or colony merging. However, this method is inaccurate if small sample sizes are used or if the frequency of induction of altered cells is very large, generally >10% (9).

The frequency of induction of EG variants of RTE cells by MNNG was not significantly different when calculated by either of these 2 methods (Table 1). This consistency in the calculated frequency of transformation using either method strengthens the reliability of our measurement of the frequencies of induction of EG variants. In addition, it provides alternative methods of calculating transformation frequencies depending on the size of the experiment and the individual experimental needs.

It is interesting to compare the transformation frequency of RTE cells to the frequency of early transformation changes in other cells. Treatment of Syrian hamster embryo fibroblasts (5) with a dose of MNNG which yielded a level of survival (50%) similar to that observed for RTE cells (53%) resulted in a frequency of morphological transformation (1.9%), which is similar to the frequency of the MNNG-induced transformation of RTE cells (2.9%). A preliminary report (16) indicated that the frequency of carcinogen-induced preneoplastic mouse epidermal cells on a per-cell basis was ~1%, which is also comparable to the induced frequency of EG variants of RTE cells (Table 1). Other cell transformation systems quantitate the conversion of preneoplastic cells to the neoplastic state (2) (e.g., C3H 10T ½, BALB/c 3T3, and BHK cells) and, therefore, differ from the above studies which involve the conversion of normal cells to neoplastic cells.

We attempted to determine the time required by RTE cells for the fixation and expression of carcinogen-induced events which result in the appearance of EG variants. This analysis was complicated by the fact that the selective conditions we used, i.e., feeder removal and subculturing, did not result in immediate cessation of growth of the normal RTE cells. Even though selection pressure was applied as early as Day 2 after treatment, some cells continued to divide for 4 to 7 more days. Hence, we can only estimate that the expression time required for the carcinogen-induced events in RTE cells is less than 10 days. Pai et al. (23) were able to observe population growth differences in carcinogen-treated versus control RTE cells 18 days after treatment, which also suggests an expression time similar to what we have estimated. Application of selection pressure, however, does offer the advantage of reducing spontaneous transforma-

tion. The reduction of spontaneous transformation with the application of selection pressure affecting normal cell proliferation suggests that spontaneous transformation occurs as a function of cell proliferation. This relationship is currently under investigation and may be important in respiratory carcinogenesis in particular and to carcinogenesis in general, since exposure to many environmental agents associated with the eventual development of cancer often results in cell proliferation due to tissue irritation or more specific mitogenic responses.

The one-hit mechanism and the time required for expression of the induction of the EG variant are consistent with this alteration resulting from a mutational event. However, the frequency of cell transformation in these studies is significantly higher than frequencies of gene mutations commonly observed in mammalian cells in culture. The mechanism for the high frequency of cell transformation of fibroblasts and epithelial cells in culture is unknown. At least 4 explanations (1) can be offered for these results. The high frequency of transformation may be due to: (a) a large target size for these phenotypes; (b) mutational “hot spots” in the gene (or genes) controlling these phenotypes; (c) a mutational basis other than gene mutations for these phenotypic alterations, i.e., chromosomal mutations; or (d) a nonmutational alteration in gene expression. Measurement of only the frequency of cell transformation cannot distinguish between these possibilities. However, our recent experiments with fibroblasts suggest a chromosomal mutation as the basis for cell transformation (3, 13).

The relationship between early preneoplastic changes of cells in culture and early, carcinogen-induced events in vivo is unknown, because quantitation of the frequency of these events in vivo is lacking. However, recent experiments by Nomura (22) have demonstrated a high frequency (up to 30%) of tumors in offspring following parental exposure to a variety of carcinogens. The “mutations” which predispose germ cells to tumorigenesis are similar to other accepted germ-line mutations in terms of heritability, mutagen sensitivity, pattern of stage-dependent sensitivity of the germ cells, and doubling dose following X-ray treatment. However, germ-line “tumor” mutations occurred much more frequently than did other germ-line mutations in the same animals, i.e., about 10 times higher than dominant skeletal mutations. The apparently high frequency of induction of these germ-line “tumor” mutations in vivo suggests a possible relationship between the high frequency of carcinogen-induced changes in cells in culture and the high frequency of neoplasia-related changes inducible in vivo. Any theory of carcinogenesis must provide an explanation for the high frequency of heritable changes in cells in vivo and in vitro, which result in an increased propensity of the cells to become neoplastic.

The RTE cell transformation system described here is unique and important for studies aimed at understanding the mechanism of chemical carcinogenesis. The system uses cells from an environmentally and epidemiologically relevant tissue, respiratory epithelium. The frequency of carcinogen-induced, early preneoplastic changes can be quantitated on a per-cell basis, and that frequency is high. This system will be useful for further studies on the mechanism of early changes in epithelial transformation as well as later stages in the neoplastic development of the cells.

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Fig. 1. EG variants of RTE cells. A, EG variant fixed with methanol and stained with 10% aqueous Giemsa in 35-mm-diameter dish 6 weeks after MNNG treatment. B, high magnification (x 100) photomicrograph of EG variant cells fixed and stained as above 6 weeks after MNNG treatment.

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High Frequency of Carcinogen-induced Early, Preneoplastic Changes in Rat Tracheal Epithelial Cells in Culture

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